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RESEARCH PAPER

Protection of tobacco cells from oxidative copper toxicity by catalytically active metal-binding DNA oligomers

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Abstract

The impact of copper ions on the oxidative and calcium signal transductions, leading to cell death in plant cells, have been documented. Copper induces a series of biological and chemical reactions in plant cells including the oxidative burst reflecting the production of reactive oxygen species and the stimulation of calcium channel opening allowing a transient increase in cytosolic calcium concentrations. These early events, completed within a few minutes after the contact with copper, are known to trigger the development of cell death. The effects of DNA fragments with copper-binding motifs as novel plant cell-protecting agents were assessed using cell suspension cultures of transgenic tobacco (*Nicotiana tabacum* L., cell line BY-2) expressing the aequorin gene. The addition of GC-rich double-stranded DNA fragments, prior to the addition of copper ions, effectively blocked both the copper-induced calcium influx and cell death. In addition, the DNA–Cu complex examined was shown to possess superoxide-scavenging catalytic activity, suggesting that DNA-mediated protection of the cells from copper toxicity is due to the removal of superoxide. Lastly, a possible mechanism of DNA–Cu interaction and future applications of these DNA fragments in the protection of plant roots from metal toxicity or in aid of phyto-remediation processes are discussed.

Key words: Aequorin, cell death, copper phytotoxicity, metal toxicity, nucleozyme, ROS, Z-DNA.

Introduction

In most plants, exposure to elevated concentrations of heavy metals results in growth inhibition and severe damage although a certain amount of metal ions such as of iron, cobalt, copper, manganese, molybdenum, and zinc are required by all living organisms (Dietz *et al.*, 1999). Visible symptoms of copper toxicity, such as chlorosis reflecting the loss of chlorophylls and necrotic lesions reflecting localized cell death, might largely be due to oxidative stress accompanying the direct and indirect actions of copper ions. Earlier work has suggested that treatment of living plants and tissues with excess copper (up to the sub-millimolar range) coincide with the production of, molecular responses to, and cellular damage

by, reactive oxygen species (ROS) such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$), possibly by direct electron transfer involving metal cations, the formation of catalytic complexes with natural chelating agents such as small peptides, or as a consequence of metal-mediated inhibition of metabolic reactions (Dietz *et al.*, 1999; Drążkiewicz *et al.*, 2004; Tewari *et al.*, 2006; Bona *et al.*, 2007; Gonzalez *et al.*, 2010; Yokawa *et al.*, 2011*a*).

It is well known that homeostasis in the levels of ROS and cross-talk among ROS and other signalling events are crucial to plant responses and to the adaptation to abiotic environmental factors that threaten the plants (Lin *et al.*, 2006*a*; Kadono *et al.*, 2010; Kunihiro *et al.*, 2011; Yokawa *et al.*, 2011*b*; Tran *et al.*, 2013*a*, *b*). One of the key signalling events contributing to the above cross-talk would be a transient increase in the cytosolic free calcium ion concentration ($[Ca^{2+}]_c$). The induction of cell death by oxidative stresses that accompany the generation of ROS often involves the preceding influx of Ca^{2+} into the cytosolic space via the ROS-dependent activation of calcium channels on the vacuolar and/or plasma membranes (Lin *et al.*, 2006*a*; Kadono *et al.*, 2006, 2010).

Our previous studies have revealed that various phytotoxic metal ions such as Al^{3+} and of lanthanides (Ln^{3+}) induce an acute generation of ROS such as $O_2^{\cdot-}$ by stimulating the activity (Kawano *et al.*, 2001, 2002, 2003) and expression (Kunihiro *et al.*, 2011) of plant NADPH oxidase. As a consequence, metal-dependently produced ROS stimulates the opening of ROS-responsive calcium channels in plant cells (Kawano *et al.*, 2003, 2004; Lin *et al.*, 2005). Conversely, the redox-responsive calcium channels could be blocked when Al^{3+} or Ln^{3+} (such as La^{3+}, Nd^{3+}, Eu^{3+}, Sm^{3+}, Gd^{3+} or Tb^{3+}) is present at an excess level (Kawano *et al.*, 2003; Lin *et al.*, 2006*b*).

Copper is also known to be a phytotoxic metal which induces both the oxidative burst and an increase in $[Ca^{2+}]_c$ which is eventually followed by cell death in both aquatic plants (Gonzalez *et al.*, 2010; Min *et al.*, 2013) and terrestrial plant cells such as cultured tobacco cells (Inoue *et al.*, 2005; Kagenishi *et al.*, 2009). Interestingly, the possible mechanism of acute copper action may differ from the above metals which induce $O_2^{\cdot-}$ generation via enzyme activation. According to earlier work (Chiou, 1983), copper ions damage the DNA molecules through the formation of $HO^{\bullet}$ after reacting with H_2O_2. It has been proposed that reduction by H_2O_2 of Cu(II) to Cu(I) is followed by a further reaction between Cu(I) and H_2O_2, finally releasing the most violent ROS, $HO^{\bullet}$ as below.

$$Cu(II) + H_2O_2 \longrightarrow Cu(I) + HO_2 + H^+$$

$$Cu(I) + H_2O_2 \longrightarrow Cu(II) + OH^- + HO^{\bullet}$$

Note that $O_2^{\cdot-}$ is readily derived from HO_2 depending on pH, and, therefore, copper ions could be considered as efficient inducers of $O_2^{\cdot-}$ where H_2O_2 is supplied by living cells. Furthermore, free copper ions and even the Cu derived from Cu/Zn-superoxide dismutase (SOD) catalyse the Fenton-type reactions for the production of $HO^{\bullet}$, if $O_2^{\cdot-}$, the source of H_2O_2, is provided in the presence of reducing sugars such as glucose (Kaneto *et al.*, 1994). In fact, slow but continuous production of $O_2^{\cdot-}$ reportedly occurs in dilute solutions of glucose and other reducing sugars (Kaneko *et al.*, 2006), suggesting that the addition of copper ions to the mixture of similar organic chemicals or biomolecules results in the enhanced production of violent ROS members.

Previously, monitoring of the Fenton-type reactions leading to the formation of $HO^{\bullet}$ was performed by electron spin resonance spectroscopy and this reaction was shown to proceed in tobacco BY-2 cell suspension cultures after the addition of $CuSO_4$ (Kawano and Muto, 2000). Due to the hyper-reactivity of $HO^{\bullet}$ even against water molecules, $HO^{\bullet}$ hardly migrates even a short distance in the aqueous phase, thus only the portion of $HO^{\bullet}$ generated at the site adjacent to the DNA causes or enhances the degradation of, or damage to, DNA.

According to earlier work, Cu^{2+} was shown to bind and to damage DNA (Chiou, 1983; Yokawa *et al.*, 2011*c*), especially through affinity to the guanine (G) and cytosine (C) bases at physiological pH, eventually perturbing the neighbouring base pairs of adenine (A) and thymine (T) and disrupting the double-helical structure of DNA (Tajmir-Riahi *et al.*, 1988). In addition, among three of the known biologically active forms of DNA, Z-DNA showing a non-Watson–Crick type structure (Bansal, 2003), possesses the highest affinity to the binding of Cu^{2+}, especially at G bases (Geierstanger *et al.*, 1991). Accordingly, the complex between Cu and Z-DNA domains readily results in damage to the DNA molecules (Geierstanger *et al.*, 1991).

The effect of a copper-binding short peptide derived from human prion protein on the protection of tobacco BY-2 cells from copper toxicity was investigated previously (Kagenishi *et al.*, 2009). An excess of peptides (*c.* 5–10 times higher concentrations compared with that of copper ions) effectively blocked both Ca^{2+} influx and cell death induced by copper, possibly due to the removal of copper.

By analogy, it was expected that Cu-binding DNA also prevents the toxicity of copper in plant cells. Since the major targets of copper-dependent damage to DNA are the AT-base pairs, the proposed model DNA should be rich in GC-base pairs. The use of a CGCGCG DNA hexamer, one of the minimal GC-rich Z-DNAs (Ban *et al.*, 1996), is proposed here as one of the novel anti-copper molecules active in plant cell protection against the toxicity of copper. In the present study, we examined the effect of copper-binding DNA oligomers against the toxic actions of copper we examined by focusing on the Cu-responsive calcium signalling and cell death responses in suspension-cultured tobacco BY-2 cells expressing the Ca^{2+}-sensitive luminescent protein aequorin. In addition, the removal of copper-dependently formed $O_2^{\cdot-}$ by the DNA–Cu complex is discussed as the Cu-bound form of the CGCGCG DNA hexamer duplex is viewed as a novel antioxidant molecule with SOD-like catalytic activity.

Materials and methods

Plant material

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2; cell line, BY-2) expressing apoaequorin exclusively in the cytosol were propagated as previously reported by Takahashi *et al.*

(1997). Briefly, the culture was maintained in Murashige–Skoog (MS) liquid medium (pH 5.8) containing 0.2 μg ml^{-1} 2,4-dichlorophenoxy acetic acid at 23 °C with shaking on a gyratory shaker in darkness and sub-cultured weekly with 3% (v/v) inoculum. Confluent cultures, sub-cultured at 1 week intervals, were used to inoculate the fresh MS liquid medium and were then cultured for 5 d. These 5-d-old cultures were harvested and used for the experiments. For preliminary study, a redox-sensitive cell line of tobacco (Bel-W3) was also used.

Coelenterazine

Coelenterazine, a luminophore required for the reconstitution of aequorin from apoaequorin (Shimomura and Johnson, 1978), was chemically synthesized by the group of Professor Isobe, Nagoya University, Nagoya, Japan (Isobe *et al.*, 1994).

DNA oligomers

DNA oligomers, namely, CGCGCG, ATATAT, GGGGGG, and CCCCCC were synthesized (each 4 mg) on demand at Genenet Co. Ltd. (Fukuoka, Japan).

Treatments

$CuSO_4$ was first dissolved in water. The copper solution (10 μl) was added (0.03–10.0 mM, final concentration) to the cells suspended in MS medium (190 μl). When required, DNA oligomers were mixed with the copper solution prior to addition to the cell suspension.

Determination of cell death

Copper-induced cell death in the cell-suspension culture was allowed to develop in the presence of the vital staining dye, Evans blue (0.1%, w/v). The dye was added to the cell suspension culture, 10 min after the application of copper ions. The cells were further incubated for 1 h to develop cell death fully as described by Kadono *et al.* (2006). After terminating the staining process by washing, the stained cells were observed under the microscope. For statistical analyses, four different digital images of cells under the microscope (each covering over 100 cells to be counted) were acquired and stained cells were counted.

Monitoring of $[Ca^{2+}]_c$

The changes in $[Ca^{2+}]_c$ were monitored by Ca^{2+}-dependent emission of blue light from aequorin as previously described by Kawano *et al.* (1998). The active form of aequorin was reconstituted by the addition of 1 μM coelenterazine to the apoaequorin-expressing tobacco cells, 8 h prior to the measurements of $[Ca^{2+}]_c$. The aequorin luminescence was measured using a photometer (Luminescensor PSN AB-2200-R, Atto Corp., Tokyo, Japan).

Detection of O_2^-

For the detection of the O_2^- generated and/or maintained in the reaction mixture, an O_2^--specific chemiluminescence probe, *Cypridina* luciferin analogue (CLA; 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazin-3-one), was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Generation of O_2^- was monitored by the chemiluminescence of CLA with a photometer (Luminescensor PSN AB-2200-R, Atto, Tokyo), and the yield of chemiluminescence was expressed as relative luminescence units (rlu), as previously described by Kawano *et al.* (1998). CLA is a specific probe for O_2^- (and singlet oxygen to a lesser extent), but not for other ROS members (Nakano *et al.*, 1986).

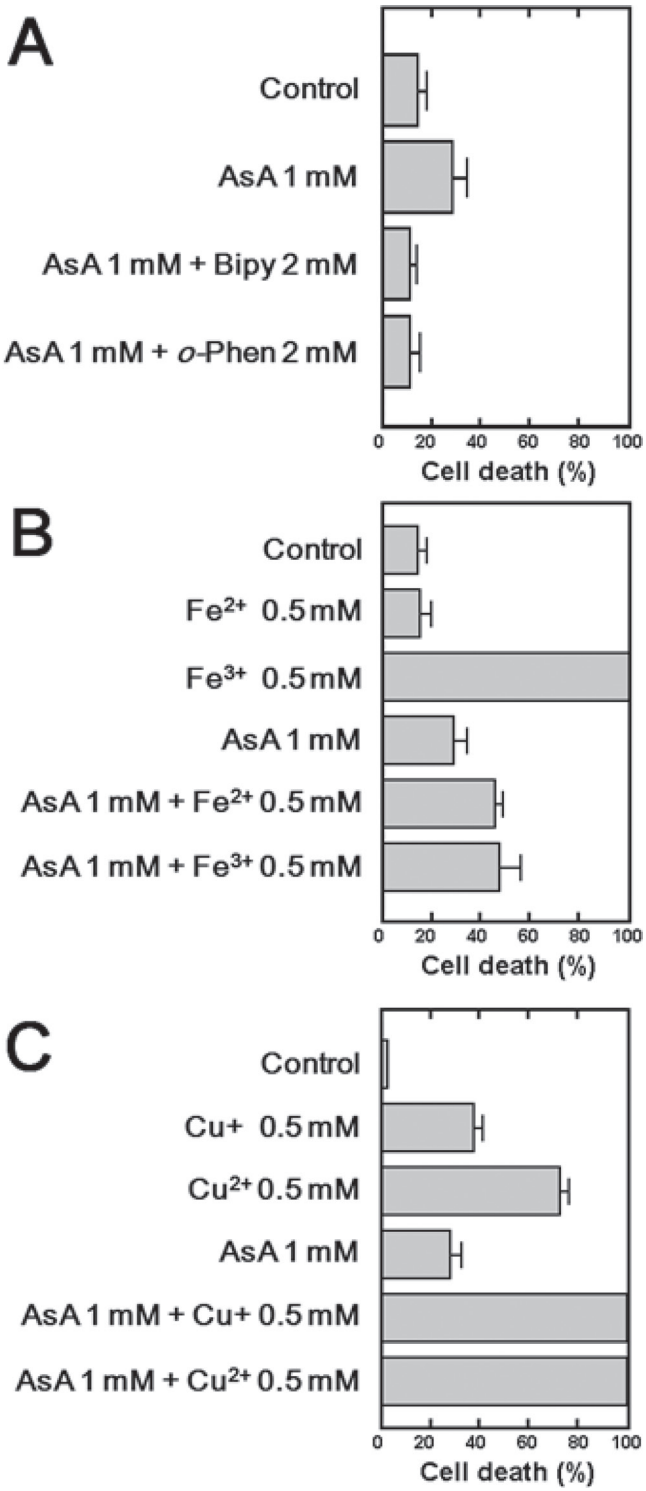

Fig. 1. Preliminary study assessing the toxic impacts of cupric, cuprous, ferric, and ferrous ions in a redox-sensitive tobacco cell line. (A) Ascorbate toxicity involving the ions of copper and iron. (B) Effects of ferric and ferrous ions on the induction of cell death. (C) Effects of cupric and cuprous ions on the induction of cell death. To elucidate the involvements of redox cycling in Fe/Cu toxicity, 1 mM ascorbic acid (AsA) was used as both a reducing agent and an antioxidant. 2 mM 2′,2-bipyridyl (Bipy) and *o*-phenanthroline (*o*-phen) were used as Fe and Cu chelators, respectively.

Generation of $O_2^{\cdot-}$

Three different sources of $O_2^{\cdot-}$ were used for assessing the catalytic activity of DNA oligomers (see cases 1, 2, and 3). For manifesting the short pulse of $O_2^{\cdot-}$ increase, potassium superoxide (KO_2) dissolved in dry DMSO was used as the standard source of $O_2^{\cdot-}$ and injected into a potassium phosphate buffered (50 mM, pH 7.0) DNA–Cu mixture in the photometric apparatus (case 1). For reproducing the continuous but slow release of $O_2^{\cdot-}$ in the presence of copper ions and H_2O_2, a known path for Cu^{2+}-mediated conversion of H_2O_2 to $O_2^{\cdot-}$ via $HO_2^{\cdot}$ was demonstrated by injecting H_2O_2 into the Cu-containing reaction mixture in the photometric apparatus (case 2). For the preparation of a sono-photocatalytically activated $O_2^{\cdot-}$-rich medium, a titania-coated alumina fibre-equipped ultraviolet and ultrasonic wave-driven sono-photocatalytic apparatus designated as Ex-PCAW (WIPO No: WO10/032765), K2R Inc. (Kitakyushu, Japan) was used. It comprised a UV-A emitting bulb, ultrasonic wave-generating devices, sheets of titania-coated fibres, a pumping system, and dissolved O_2 supply systems. With this apparatus, $O_2^{\cdot-}$ concentration in the reaction mixture could be maintained at a desired range between 0.05 µM and 1 µM. DNA-mediated $O_2^{\cdot-}$ removal was assessed under different $O_2^{\cdot-}$ concentrations (case 3).

Tetrazolium-based SOD assay kit

In addition to the three cases of $O_2^{\cdot-}$ removal by the DNA–Cu complex, a commercial SOD assay kit employing xanthine oxidase (XO) and water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulpho-phenyl)-2H-tetrazolium, monosodium salt), was obtained from Dojindo Laboratories (Kumamoto, Japan). The XO reaction and formation of formazan were performed as indicated by the vendor's instruction. Spectroscopic assessment of the SOD-like action (a decrease in absorption corresponding to formazan) by the DNA–Cu complex was monitored with a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan).

Results and discussion

Toxicity of cupric and ferric ions: a preliminary study

It is well known that copper-dependent oxidative stress is not prevented by common reducing agents such as ascorbate (AsA), glutathione, and other thiols, since these reducing agents contribute to reproduce the Fenton-active catalyst Cu^{+} from Cu^{2+} (Yokawa *et al.*, 2011c; Kawano *et al.*, 2013). To confirm the above notions in tobacco cells, the impact of copper and iron ions was tested in combination with AsA which is used as the model reducing agent (Fig. 1). An excess of AsA added to the cell suspensions slightly increased the level of cell death (Fig. 1A). This increase was shown to be mediated by two of the known Fenton catalysts endogenously present in plant cells as the chelators of Cu^{+} and Fe^{2+} effectively blocked the cell-killing action of AsA (Fig. 1A). Therefore, one might predict that the toxicity of ferrous ions is higher than that of ferric ions and, similarly, a higher toxicity for cuprous ions than cupric ions is expected based on the performance of the above ions in Fenton-type reactions converting H_2O_2 to $HO^{\cdot}$. Interestingly, additions of ferric and cupric ions were shown to be much more highly toxic compared with the actions of ferrous and cuprous ions (Fig. 1B, C). Therefore, the overall roles must be considered of ferric/ferrous and cupric/cuprous

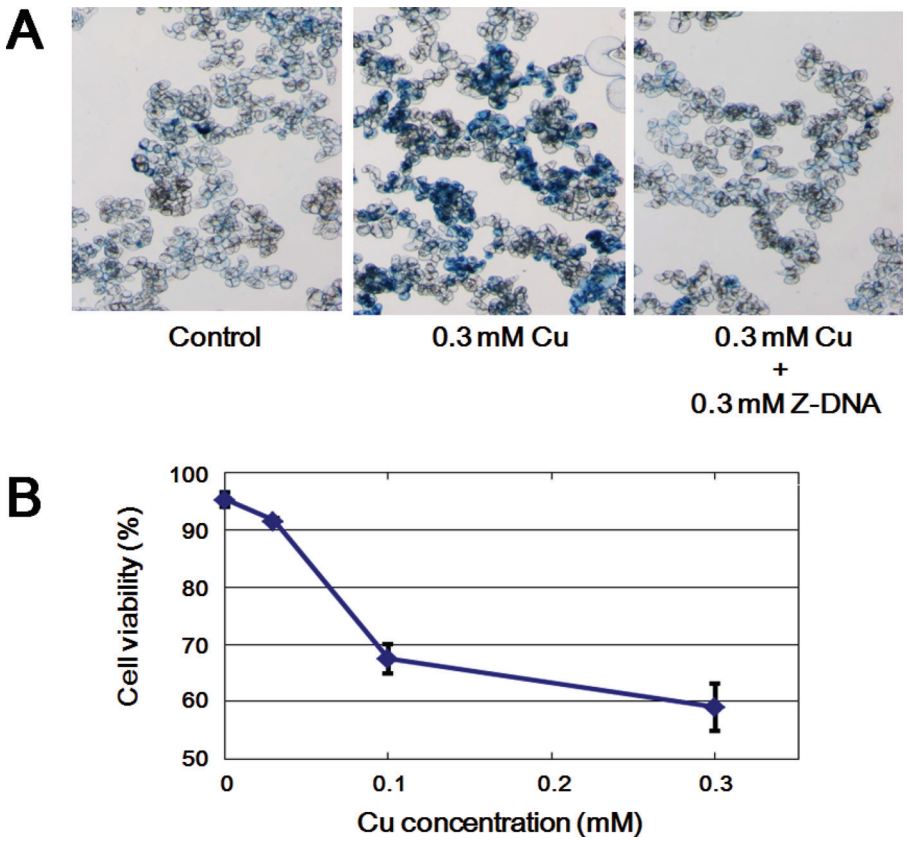

Fig. 2. Visualization and quantification of copper-induced cell death in tobacco BY-2 cells. (A) Vital staining revealed the cupper-induced cell death and its inhibition by CG-repeated double-stranded DNA hexamer (CGCGCG). (B) Effect of copper concentration on the induction of cell death. Error bars represent S.D. (n=4). (This figure is available in colour at *JXB* online.)

redox cycles involving a series of redox interactions with various molecules covering both the oxidants and reductants, finally determining the status of redox stress and cellular damage. It is noteworthy that the severely toxic action of ferrous ions can be partially blocked by an excess of AsA, while the toxicity of cupric ions has a tendency to be highly enhanced in the presence of AsA (Fig. 1C). Hereafter, the focus was on the toxicity of cupric ions only, since cupric ions were shown to be more toxic than cuprous ions and more difficult to detoxify than ferric ions by the use of reducing agents.

Prevention of Cu-induced cell death by DNA oligomers

As previously demonstrated, the addition of $CuSO_4$ resulted in acute cell death in tobacco BY-2 cells. Following the early events represented by ROS production and the ROS-mediated increase in $[Ca^{2+}]_c$ induced by Cu treatment, which are probably completed within 5–10 min following the application of Cu, the induction of cell death occurred during the additional 1 h of static incubation. The dead cells, stained by Evans blue, were photographed under a microscope and counted in order to assess cell viability (Fig. 2A). Data suggested that copper induces cell death in a dose-dependent manner (Fig. 2B).

Preliminary data (Fig. 1) strongly suggested that the use of known reducing agents, often considered to be effective in preventing the oxidative burst, is not a wise choice to prevent copper-mediated toxicity in tobacco cells. In turn, the likely approach, by analogy to previous work with a Cu-binding peptide (Kagenishi *et al.*, 2009, 2011) is the removal or trapping of redox active copper ions by macromolecules. DNA hexamers harbouring a Cu-binding motif found in Z-DNA was chosen as a model molecule here.

As expected, the addition to the cell suspension culture of the model GC-rich DNA hexamer with minimal Z-DNA structure, CGCGCG, prior to the addition of copper, effectively blocked the induction of cell death by copper (Fig. 3). On the other hand, the addition of a model AT-rich DNA hexamer (ATATAT) showed no protective action against the toxicity of copper in tobacco BY-2 cells (Fig. 3B). Both of the model DNA samples were designed to form the duplex structure (thus double-stranded). The above data strongly suggested that detoxification of

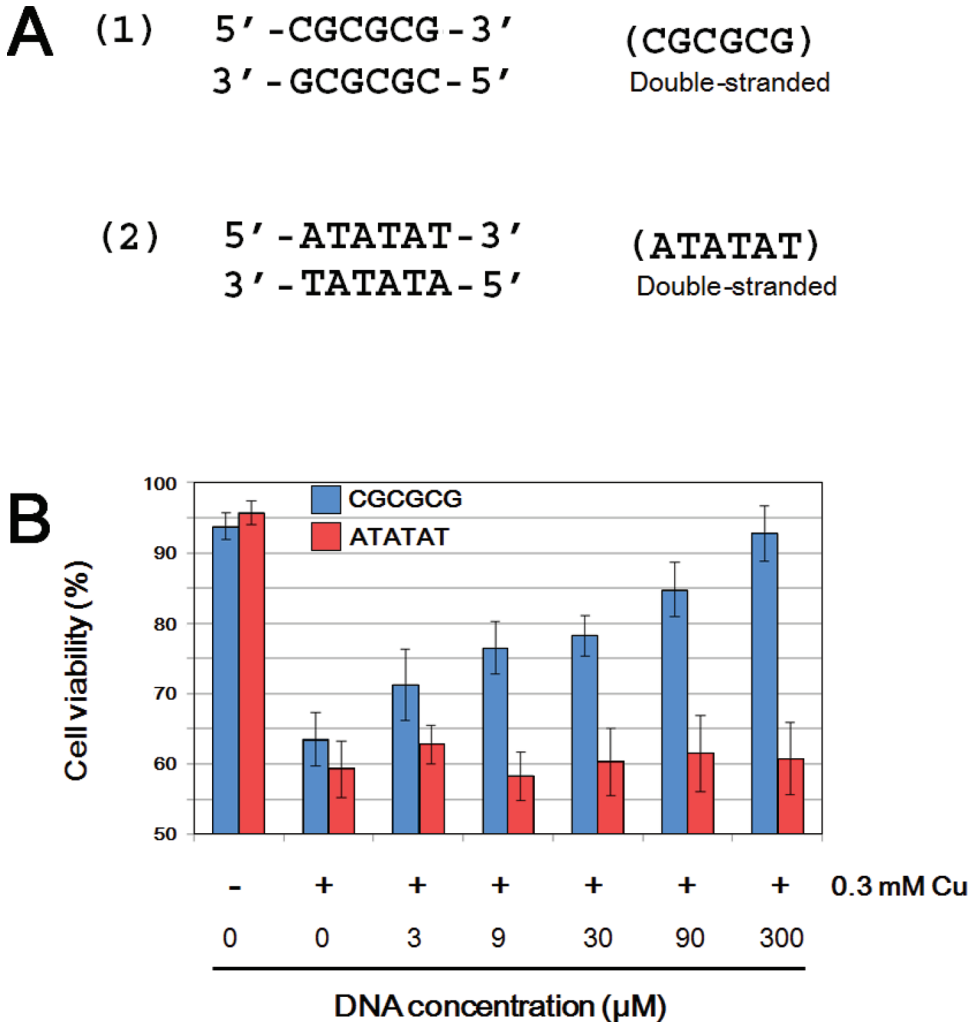

Fig. 3. Protection of tobacco BY-2 cells from copper-induced cell death by Z-DNA. (A) Model GC-rich and AT-rich double-stranded DNA oligomers used. (B) Effect of AT-repeated (ATATAT) and CG-repeated (CGCGCG) double-stranded DNA hexamers on cell death induction by 0.3 mM $CuSO_4$. Error bars represent S.D. (n=4). (This figure is available in colour at *JXB* online.)

copper ions requires the presence of GC base pairs in the double-stranded DNA.

To test the hypothesis that GC pairs in the double-stranded DNA are essential for the protection of cells from copper-induced cell death, the effects were compared of four different combinations of DNA hexamers consisting of either C, G, or both (Fig. 4). Cell death induction by 0.3 mM $CuSO_4$ was shown to be inhibited by two different treatments with double-stranded GC-rich DNAs, namely a CGCGCG sequence and a 1:1 mixture of a CCCCCC sequence and a GGGGGG sequence. On the other hand, the addition of a CCCCCC or a GGGGGG sequence alone showed no inhibitory action against copper-induced toxicity, confirming that single-stranded DNA fragments are inactive in the detoxification of copper.

Prevention of Cu-induced Ca^{2+}influx by DNA oligomers

Aequorin was reconstituted from apo-protein expressed in the cytosol of tobacco BY-2 cells by adding coelenterazine to the culture 8 h prior to the experiments. The addition of $CuSO_4$ (from 0.03 mM to 10 mM) resulted in a rapid and transient increase in aequorin luminescence reflecting the acute increase in $[Ca^{2+}]_c$ in a dose-dependent manner (Fig. 5A, B).

The Cu (0.3 mM)-induced increase in $[Ca^{2+}]_c$ was significantly lowered by a 0.3 mM GCGCGC sequence (Fig. 5). On the other hand, ATATAT did not prevent Cu-induced Ca^{2+} influx (Fig. 6). These data form the first demonstration that an exogenously applied non-coding Z-DNA fragment can significantly modify a signalling event in the plant system.

Removal of O_2^- by the DNA–Cu complex

Up to now, facts have been accumulated that a minimal Z-DNA sequence prevents copper toxicity in tobacco cells. However, the mechanism is still obscure since minor doses, lower than that of the applied copper, also showed a significant effect in the protection of cells. For instance, Cu-binding peptides reportedly failed to prevent copper-induced cell death and stimulation of calcium influx when the ratio between peptides and copper ions was lower than 3:1 (Kagenishi *et al.*, 2009). Thus, it is natural to speculate that there is alternative mechanism in addition to merely trapping the toxic ions.

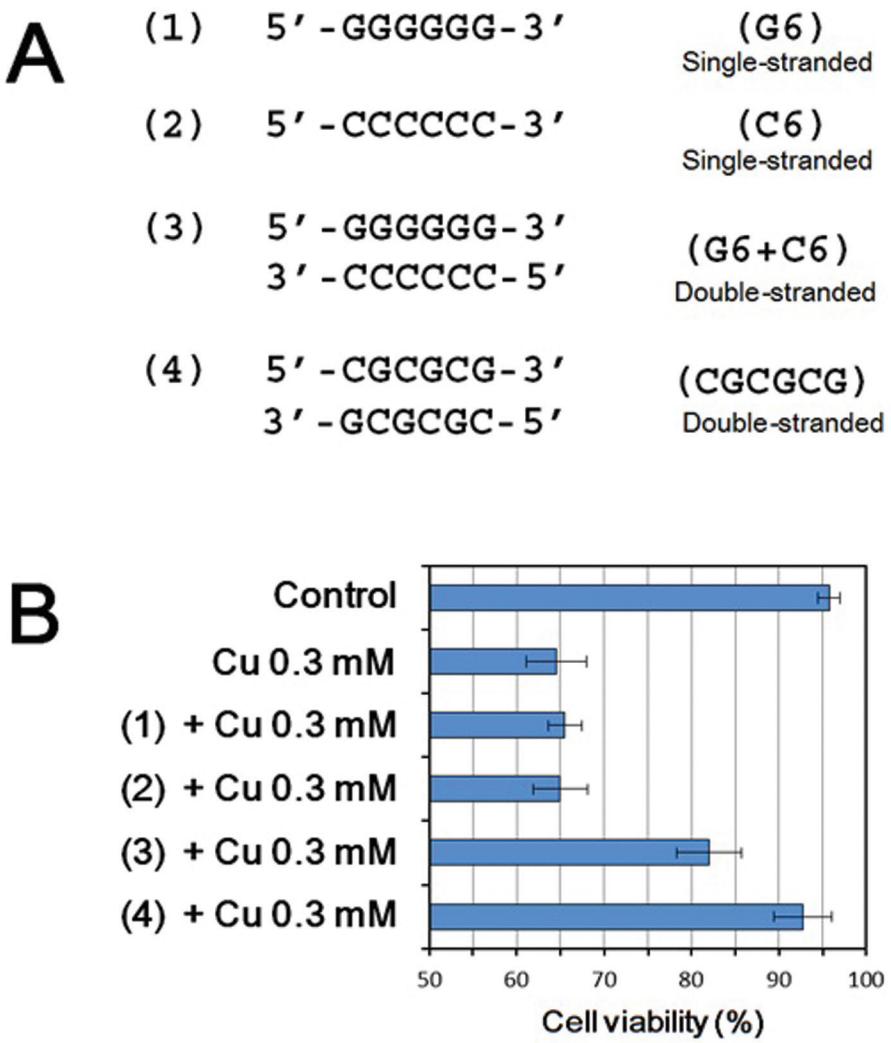

Fig. 4. Structural requirement of GC-rich DNA hexamers for inhibition of copper toxicity in tobacco BY-2 cells. Concentration of DNA was fixed at 0.3 mM, regardless of being single- and double-stranded forms. (A) Combinations of GC-rich model DNA oligomers used. (B) Effect of single-stranded and double-stranded GC-rich DNA hexamers on cell death induction by 0.3 mM $CuSO_4$. Error bars represent S.D. (n=4).

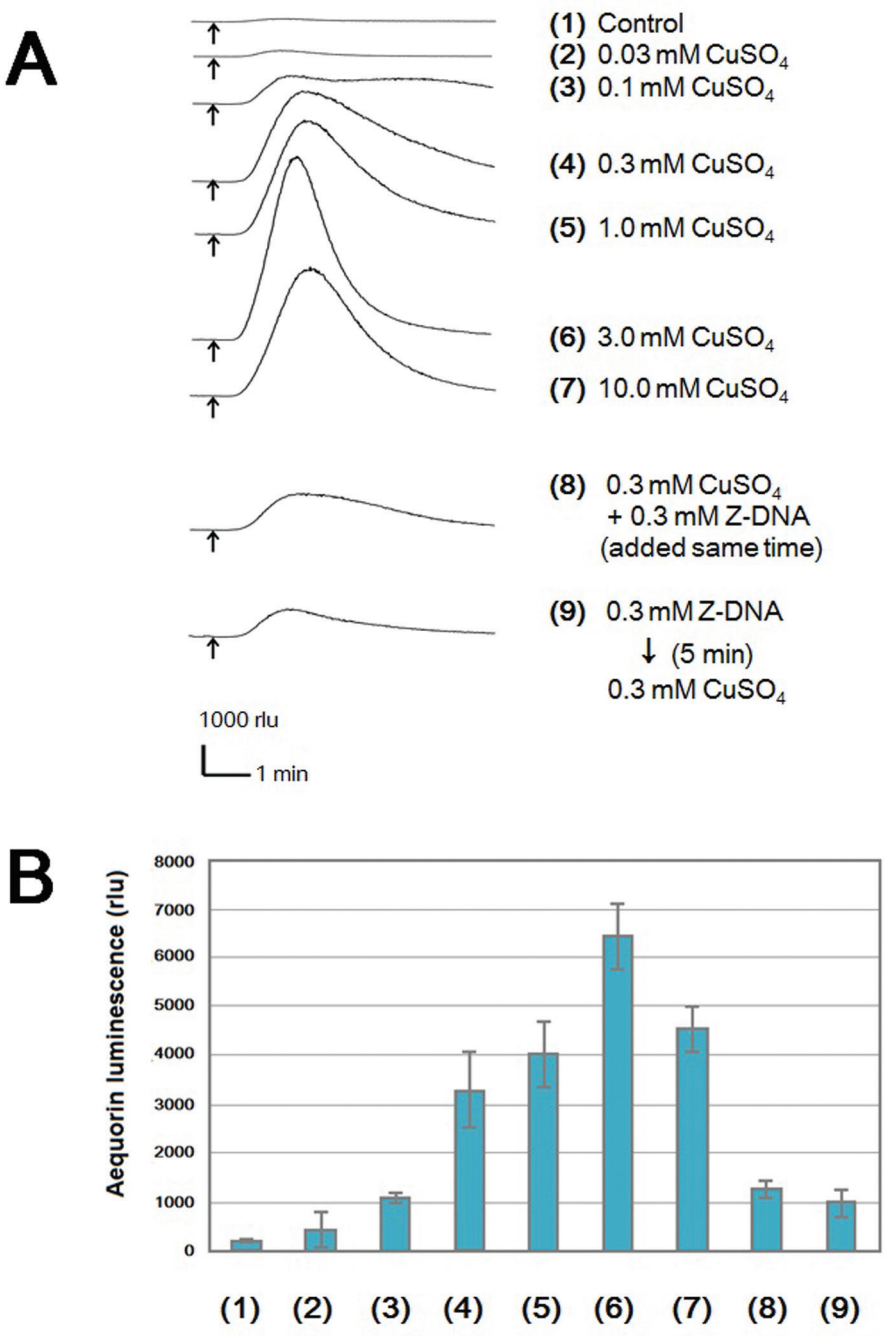

Fig. 5. Cu-induced $[Ca^{2+}]_c$ elevation and its inhibition by the addition of the Cu-binding DNA hexamer in tobacco BY-2 cells expressing aequorin. (A) Typical traces of the Cu-induced increase in aequorin luminescence. (B) Effect of $CuSO_4$ concentration ranging from 0.03 mM to 10 mM on the induction of $[Ca^{2+}]_c$ increase. Effect of the CGCGCG DNA hexamer was examined at two different times, namely, 5 min prior to and at the same time as the addition of 0.3 mM $CuSO_4$. Error bars represent S.D. (n=4).

Our previous study revealed that the acute increase in $[Ca^{2+}]_c$ is due to induced Ca^{2+} influx via stimulation of the Ca^{2+}-permeable ion channels by Cu-dependently produced ROS, since both ROS scavengers and Ca^{2+} channel blockers effectively lowered the level of $[Ca^{2+}]_c$ increase induced by copper ions (Kagenishi *et al.*, 2009). It is, therefore, highly likely that the removal of ROS could be one of the key steps required for the prevention of copper toxicity leading to programmed cell death via the stimulation of the calcium signalling mechanism.

Lastly, the possibility was tested that complexes formed between the GC-rich double-stranded DNA hexamers are catalytically capable of removing toxic ROS generated in the presence of copper ions through the Cu^+/Cu^{2+} redox cycle. There was a need to find out if the mixture of Cu^{2+} and CGCGCG DNA hexamer removes or degrades the $O_2^{\cdot-}$. Three different sources of $O_2^{\cdot-}$ were used here for assessing the catalytic activity of DNA oligomers.

Case 1: For manifesting the short pulse of $O_2^{\cdot-}$ increase, KO_2 dissolved in dry DMSO was injected into the reaction

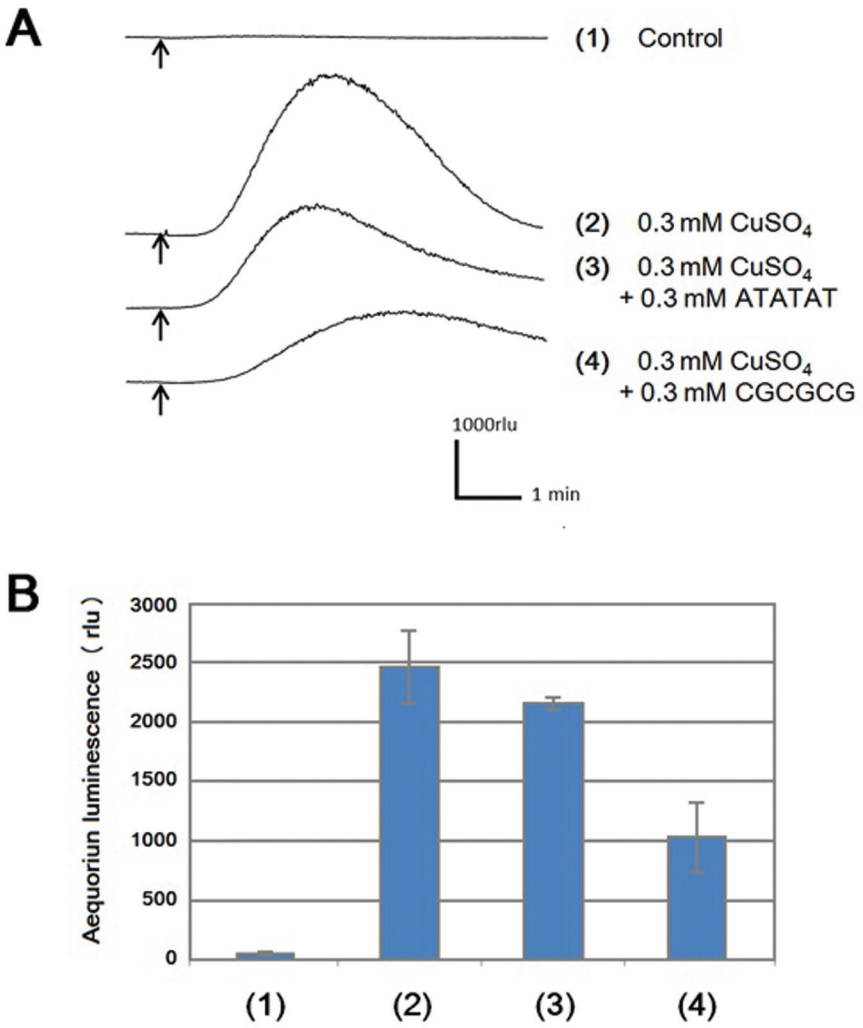

Fig. 6. Comparison of AT-repeated and CG-repeated DNA hexamers in the inhibition of the copper-induced increase in aequorin luminescence in tobacco BY-2 cells suspension culture. (A) Typical traces of a copper-induced increase in aequorin luminescence and its inhibition by DNA oligomers. (1) Water control, (2) addition of $CuSO_4$ only (0.3 mM, final concentration), (3) mixture of CGCGCG (0.3 mM, final concentration) and $CuSO_4$ (0.3 mM, final concentration), (4) mixture of ATATAT (0.3 mM, final concentration) and $CuSO_4$ (0.3 mM, final concentration). (B) Quantification of the action of two model DNA oligomers (CGCGCG, ATATAT) against the Cu-induced Ca^{2+} influx in tobacco BY-2 cells. Concentrations of both DNA and copper were identical with (A). Error bars represent S.D. (n=4).

mixture containing the CGCGCG DNA hexamer and $CuSO_4$ (Fig. 7A). Following injection of 5 µM KO_2 to the copper solution (10 µM $CuSO_4$), a spike of CLA chemiluminescence reflecting the release of $O_2^{\cdot-}$ was observed. In the presence of 1:1 v/v copper and CGCGCG DNA hexamer (each 10 µM), the KO_2-induced increase in CLA chemiluminescence was shown to be lowered. In the absence of metal, DNA (CGCGCG) added alone showed no scavenging action at any concentration tested (between 10 and 250 µM) while copper alone only started to decay $O_2^{\cdot-}$ at higher concentrations (100–250 µM). Since the DNA–Cu complex showed detectable activity at lower concentrations, the catalytic $O_2^{\cdot-}$-removing action of Cu could be enhanced in the DNA-bound form.

Kinetic analysis with the Lineweaver–Burk (double-reciprocal) plot further evaluated the V_{max} for the $O_2^{\cdot-}$-degrading action of the DNA–Cu complex, performed in the presence of various concentrations of KO_2 (used as model substrate), to be 1.675 nmol mg^{-1} DNA s^{-1} (Fig. 7A, inset).

Case 2: For reproducing the continuous but slow release of $O_2^{\cdot-}$ from H_2O_2 with the aid of copper ions, a known path for Cu^{2+}-mediated conversion of H_2O_2 to $O_2^{\cdot-}$ via HO_2, was demonstrated by injecting H_2O_2 into the Cu-containing reaction mixture in the photometric apparatus (Fig. 7B). The conversion of H_2O_2 into $O_2^{\cdot-}$ is catalyzed by free copper and thus, no $O_2^{\cdot-}$ is produced in the absence of copper (CGCGCG alone), and copper-dependently formed $O_2^{\cdot-}$ is probably removed by the DNA–Cu complex. Again, the data are supporting that copper-mediated $O_2^{\cdot-}$ production was inhibited by the CGCGCG DNA hexamer.

Case 3: For the ease of controlled kinetic analysis, an $O_2^{\cdot-}$-rich medium was prepared via a sono-photocatalytic reaction using the titania-coated alumina fibre-equipped, ultra violet-irradiated, and ultrasonic wave-driven

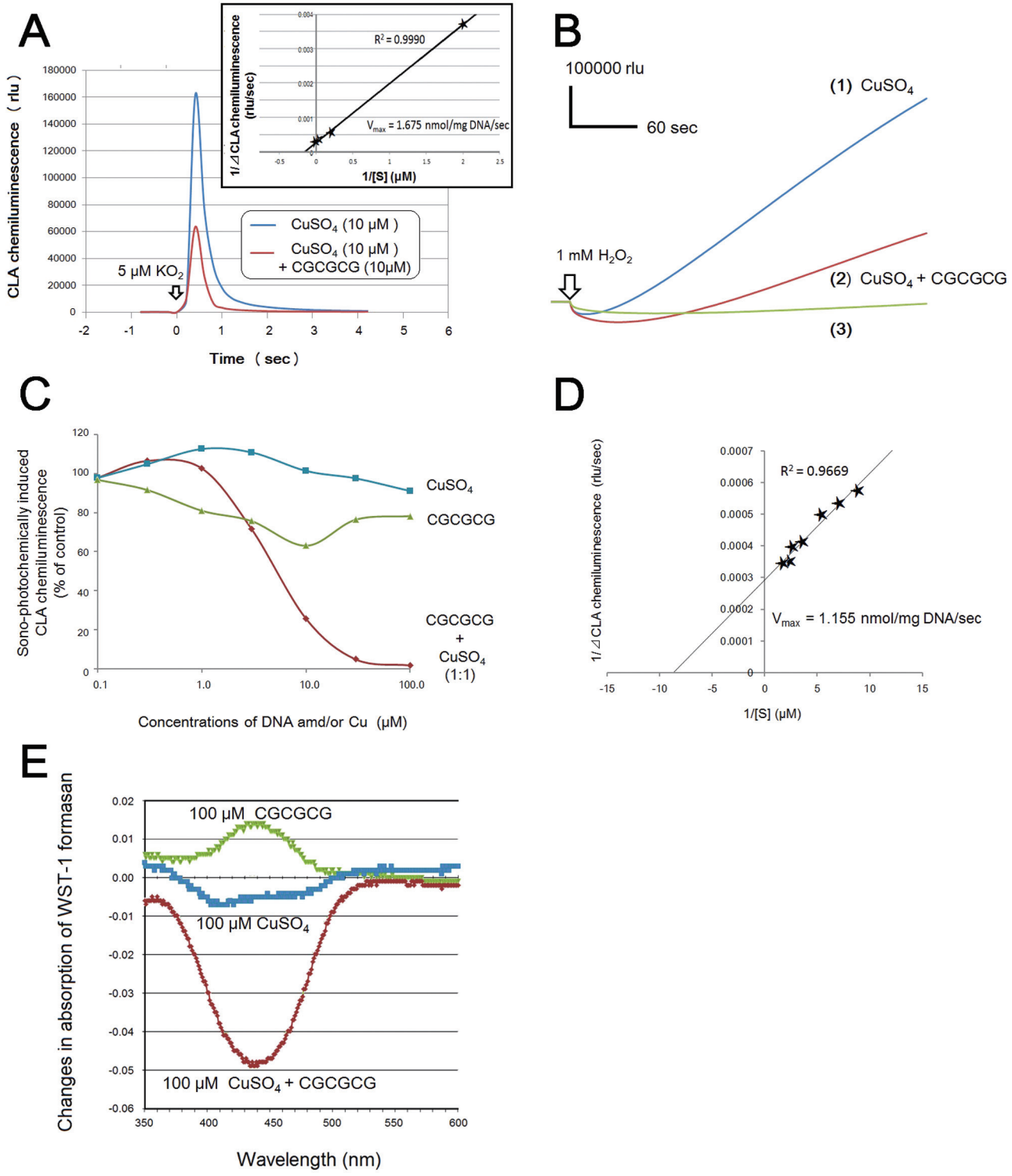

Fig. 7. Removal of $O_2^{\cdot-}$ by Cu-loaded CGCGCG DNA oligomers. (A) CLA-chemiluminescence reflecting the release of $O_2^{\cdot-}$ after injection of KO_2 into the reaction mixture containing copper and CGCGCG DNA oligomers. Inset, kinetic analysis (Lineweaver–Burk plot) of the $O_2^{\cdot-}$-removing activity in the DNA–Cu complex. (B) H_2O_2-dependent $O_2^{\cdot-}$ production in copper solution. 1 mM H_2O_2 was injected into the reaction mixture (pH 7.0, 50 mM K-phosphate) containing 25 μM $CuSO_4$ and/or 25 μM CGCGCG DNA hexamer. Data shown represent the mean of four replications. (C) Effect of the DNA–Cu complex concentration on the removal of sono-photocatalytically produced $O_2^{\cdot-}$. (D) Kinetic analysis of the $O_2^{\cdot-}$-removing activity in a DNA–Cu complex in the sono-photochemical apparatus. (E) Inhibition of XO-dependent WST-1 formazan formation by DNA–Cu complex. Difference in absorption between the control and three distinct treatments (either or both of $CuSO_4$ and CGCGCG DNA, each 100 μM) are compared. (This figure is available in colour at *JXB* online.)

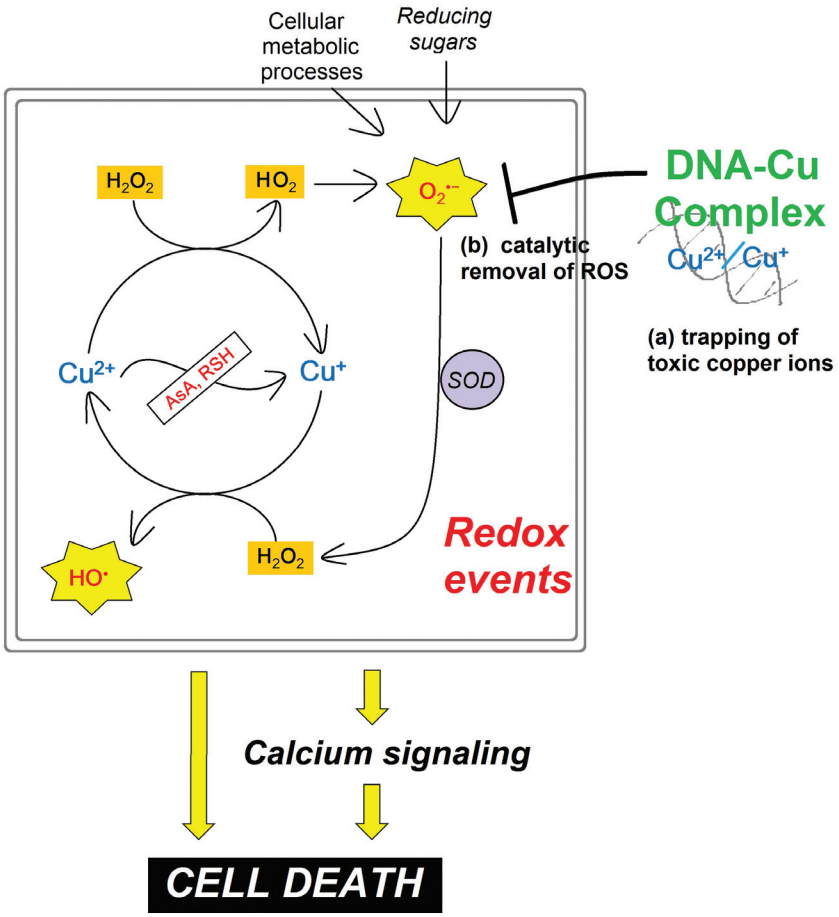

Fig. 8. A model mechanism for DNA oligomer-mediated protection of tobacco cells from copper toxicity. (This figure is available in colour at *JXB* online.)

sono-photocatalytic apparatus as described in the Materials and methods. With this apparatus, $O_2^{\cdot-}$ concentration in the reaction mixture could be maintained at a desired range between 0.05 and 1 µM. Activity of the DNA–Cu complex for the removal of $O_2^{\cdot-}$ was assessed under controlled $O_2^{\cdot-}$ concentrations. When the concentration of sono-photocatalytically generated $O_2^{\cdot-}$ was controlled to be at *c.* 0.5 µM, the yield of CLA chemiluminescence reflecting the presence of $O_2^{\cdot-}$ was lowered by the DNA (CGCGCG)–Cu complex in a dose-dependent manner (Fig. 7C). Contrary to case 2, $O_2^{\cdot-}$ is continuously provided by the sono-photocatalytic system and, thus, in the absence of any element, a high level of chemiluminescence in the buffered water was continuously observed and this level is expressed as 100% of relative CLA chemiluminescence yield (Fig. 7C). The data with CGCGCG alone and copper alone clearly suggested that removal of $O_2^{\cdot-}$ (lowering of CLA chemiluminescence) requires the presence of both CGCGCG and copper at the same time.

The range of DNA–Cu linearly lowering the level of sono-photocatalytically generated $O_2^{\cdot-}$ were shown to be equivalent to the range of Cu/Zn-SOD concentrations between *c.* 200 and 2000 units ml^{-1} (data not shown).

By modulating the levels of sono-photocatalytically released $O_2^{\cdot-}$, the kinetic analysis could be performed with the Lineweaver–Burk plot to determine graphically the V_{max} for the $O_2^{\cdot-}$-degrading action of the DNA–Cu complex to be 1.155 nmol mg^{-1} DNA s^{-1} (Fig. 7D).

Case 4: Combination of tetrazolium salts and xanthine oxidase (XO) is often employed for visualizing the removal of $O_2^{\cdot-}$ by SOD (Camejo *et al.*, 2012). To clarify that CGCGCG–Cu complex, but not CGCGCG DNA alone nor copper alone, possesses the SOD-like action for the removal of $O_2^{\cdot-}$, a test has also been conducted using a commercial SOD assay kit using XO and the water-soluble tetrazolium salt known as WST-1 which produces a water-soluble formazan dye upon reduction with $O_2^{\cdot-}$. An increase in WST-1 formazan, reflecting the XO-dependent production of $O_2^{\cdot-}$, was shown to be inhibited in the presence of the DNA–Cu complex but not in the presence of DNA alone or copper alone (Fig. 7E).

Mechanism of DNA action

To our knowledge, the oxidative toxicity by copper ions is highly complex involving many interacting agents such as ROS and reducing chemicals, and concomitant signalling events activating the programmed cell death mechanism (Kawano *et al.*, 2013). Here, the likely modes and putative roles of exogenously applied DNA oligomers with Cu-binding motifs are summarized and illustrated (Fig. 8). The primary role of such DNA, chiefly the Z-DNA minimal

sequences, is the trapping of toxic copper ions. This may be the basal mechanism for protecting the cells from the toxicity of copper ions, similar to the reported actions of Cu-binding small peptides. Furthermore, upon binding to Cu, the model DNA hexamer (CGCGCG) showed catalytic activity for the removal of $O_2^{\cdot-}$. Taken together, by interfering with the early events in Cu-mediated redox-chain reactions, Cu-binding DNA might prevent the biochemical and signalling events afterwards, finally preventing the onset of cell death.

The above studies are indicative of the hidden fact that the Cu-binding GC-rich DNA fragments may exhibit a novel function upon loading of Cu and such function may be related to the removal of ROS produced by free Cu, thus applicable for the protection of plant cells from copper toxicity. As the V_{max} values obtained from independent assays fell in a similar range (Fig. 7A, D), it can be concluded that GC-rich double-stranded DNA has solid activity to form some catalytic complex for the removal of $O_2^{\cdot-}$. This molecule could be a good model for designing some novel inorganic element-centred artificial nucleozymes.

The likely models of Cu-complex-mediated degradation of $O_2^{\cdot-}$ should refer to the mode of action of Cu/Zn-SOD. However, further examination of this hypothesis is required in future experiments and this process may allow us to design better genetic or biochemical agents for the protection of plant cells from metal toxicity.

Since the agent tested here is a DNA fragment, genetic modification of plants for the over-production and excretion of DNA fragments is one of the possible choices to minimize the phytotoxicities of various metals in future environments.

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References

Ban C, Ramakrishnan B, Sundaralingam M. 1996. Crystal structure of the self-complementary 5′-purine start decamer d(GCGCGCGCGC) in the Z-DNA conformation. I. *Biophysical Journal* **71,** 1215–1221.

Bansal M. 2003. DNA structure: revising the Watson–Crick double helix. *Current Science* **85,** 1556–1563.

Bona E, Marsano F, Cavaletto M, Berta G. 2007. Proteomic characterization of copper stress response in *Cannabis sativa* roots. *Proteomics* **7,** 1121–1130.

Camejo D, Martí MC, Olmos E, Torres W, Sevilla F, Jiménez A. 2012. Oligogalacturonides stimulate antioxidant system in alfalfa roots. *Biologia Plantarum* **56,** 537–544

Chiou SH. 1983. DNA- and protein-scission activities of ascorbate in the presence of copper ion and a copper–peptide complex. *Journal of Biochemistry* **94,** 1259–1267.

Dietz KJ, Baier M, Krämer U. 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad MNV, Hagemeyer J, eds. *Heavy metal stress in plants*. Berlin, Heidelberg, Springer-Verlag, 73–97.

Drążkiewicz M, Skórzyńska-Polit E, Krupa Z. 2004. Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. *Biometals* **17,** 379–387.

Geierstanger BH, Kagawa TF, Chen SL, Quigley GJ, Ho PS. 1991. Base-specific binding of copper(II) to Z-DNA. The 1.3-Å single crystal structure of d(m^5CGUAm^5CG) in the presence of $CuCl_2$. *Journal of Biological Chemistry* **266,** 20185–20191.

Gonzalez A, Vera J, Castro J, Dennett G, Mellado M, Morales B, Correa JA, Moenne A. 2010. Co-occurring increases of calcium and organellar reactive oxygen species determine differential activation of antioxidant and defense enzymes in *Ulva compressa* (Chlorophyta) exposed to copper excess. *Plant, Cell & Environment* **33,** 1627–1640.

Inoue H, Kudo T, Kamada H, Kimura M, Yamaguchi I, Hamamoto H. 2005. Copper elicits an increase in cytosolic free calcium in cultured tobacco cells. *Plant Physiology and Biochemistry* **43,** 1089–1094.

Isobe M, Takahashi H, Usami K, Hattori M, Nishigohri Y. 1994. Bioluminescence mechanism on new system. *Pure and Applied Chemistry* **66,** 765–772.

Kadono T, Tran D, Errakhi R, Hiramatsu T, Meimoun P, Briand J, Iwaya-Inoue M, Kawano T, Bouteau F. 2010. Increased anion channel activity is an unavoidable event in ozone-induced programmed cell death. *PLoS ONE* **5,** e13373.

Kadono T, Yamaguchi Y, Furuichi T, Hirono M, Garrec J-P, Kawano T. 2006. Ozone-induced cell death mediated with oxidative and calcium signaling pathways in tobacco Bel-W3 and Bel-B cell suspension cultures. *Plant Signaling and Behavior* **1,** 312–322.

Kagenishi T, Yokawa K, Kuse M, Isobe M, Bouteau F, Kawano T. 2009. Prevention of copper-induced calcium influx and cell death by prion-derived peptide in suspension-cultured tobacco cells. *Zeitschrift für Naturforschung* **64c,** 411–417.

Kagenishi T, Yokawa K, Kadono T, Uezu K, Kawano T. 2011. Copper-binding peptides from human prion protein and newly designed peroxidative biocatalysts. *Zeitschrift für Naturforschung* **66c,** 182–190.

Kaneko E, Kuwano N, Matsuya H, Suzuki N, Yoda B. 2006. Generation of superoxide in the glucose solutions of pathophysiological concentrations determined by the chemiluminescence of a *Cypridina* luciferin analogue, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one. *ITE Letters on Batteries, New Technologies and Medicine* **7,** 66–68.

Kaneto H, Fujii J, Suzuki K, Kasai H, Kawamori R, Kamada T, Taniguchi N. 1994. DNA cleavage induced by glycation of Cu,Zn-superoxide dismutase. *Biochemical Journal* **304,** 219–225.

Kawano T, Hiramatsu T, Bouteau F. 2013. Signaling role of salicylic acid in abiotic stress responses in plants. In: Hayat S, Ahmad A, Alyemeni MN, eds. *Salicylic acid*. Dordrecht, The Netherlands, Springer-Verlag, 249–275.

Kawano T, Kadono T, Fumoto K, Lapeyrie F, Kuse M, Isobe M, Furuichi T, Muto S. 2004. Aluminum as a specific inhibitor of plant TPC1 Ca^{2+} channels. *Biochemical and Biophysical Research Communications* **324,** 40–45.

Kawano T, Kadono T, Furuichi T, Muto S, Lapeyrie F. 2003. Aluminum-induced distortion in calcium signaling involving oxidative bursts and channel regulations in tobacco BY-2 cells. *Biochemical and Biophysical Research Communications* **308,** 35–42.

Kawano T, Kawano N, Muto S, Lapeyrie F. 2001. Cation-induced superoxide generation in tobacco cell suspension culture is dependent on ion valence. *Plant, Cell & Environment* **24,** 1235–1241.

Kawano T, Kawano N, Muto S, Lapeyrie F. 2002. Retardation and inhibition of the cation-induced superoxide generation in BY-2 tobacco cell suspension culture by Zn^{2+} and Mn^{2+}. *Physiologia Plantarum* **114,** 395–404.

Kawano T, Muto S. 2000. Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco suspension culture. *Journal of Experimental Botany* **51,** 685–693.

Kawano T, Sahashi N, Takahashi K, Uozumi N, Muto S. 1998. Salicylic acid induces extracellular generation of superoxide followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transduction. *Plant and Cell Physiology* **39,** 721–730.

Kunihiro S, Hiramatsu T, Kawano T. 2011. Involvement of salicylic acid signal transduction in aluminum-responsive oxidative burst in *Arabidopsis thaliana* cell suspension culture. *Plant Signaling and Behavior* **6,** 611–616.

Lin C, Kadono T, Suzuki T, Yoshizuka K, Furuichi T, Yokawa K, Kawano T. 2006a. Mechanism for temperature-shift-responsive acute Ca^{2+} uptake in suspension-cultured tobacco and rice cells. *Cryobiology and Cryotechnology* **52,** 83–89.

Lin C, Kadono T, Yoshizuka K, Furuichi T, Kawano T. 2006b. Effects of fifteen rare earth metals on Ca^{2+} influx in tobacco cells. *Zeitschrift für Naturforschung* **61c,** 74–80.

Lin C, Yu Y, Kadono T, *et al*. 2005. Action of aluminum, novel TPC1-type channel inhibitor, against salicylate-induced and cold shock-induced calcium influx in tobacco BY-2 cells. *Biochemical and Biophysical Research Communications* **332,** 823–830.

Min HL, Cai SJ, Rui Z, Sha S, Xie KB, Xu QS. 2013. Calcium-mediated enhancement of copper tolerance in *Elodea canadensis*. *Biologia Plantarum* **57,** 365–369.

Nakano M, Sugioka K, Ushijima Y, Goto T. 1986. Chemiluminescence probe with *Cypridina* luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo [1,2-a] pyrazin-3-one, for estimating the ability of human granulocytes to generate O_2^-. *Analytical Biochemistry* **159,** 363–369.

Shimomura O, Johnson FH. 1978. Peroxidized coelenterazine, the active group in the photoprotein aequorin. *Proceedings of the National Academy of Sciences, USA* **75,** 2611–2615.

Tajmir-Riahi HA, Langlais M, Savoie R. 1988. A laser Raman spectroscopic study of the interaction of calf-thymus DNA with Cu(II) and Pb(II) ions: metal ion binding and DNA conformational changes. *Nucleic Acids Research* **16,** 751–762.

Tewari RK, Kumar P, Sharma PN. 2006. Antioxidant responses to enhanced generation of superoxide anion radical and hydrogen peroxide in the copper-stressed mulberry plants. *Planta* **223,** 1145–1153.

Takahashi K, Isobe M, Knight MR, Trewavas AJ, Muto S. 1997. Hypoosmotic shock induces increase in cytosolic Ca^{2+} in tobacco suspension-culture cells. *Plant Physiology* **113,** 587–594.

Tran D, El-Maarouf-Bouteau H, Rossi M, Biligui B, Briand J, Kawano T, Mancuso S, Bouteau F. 2013a. Post-transcriptional regulation of GORK channels by superoxide anion contributes towards increases in outward rectifying K^+ currents. *New Phytologist* **198,** 1039–1048.

Tran D, Kadono T, Molas ML, Errakhi R, Briand J, Biligui B, Kawano T, Bouteau F. 2013b. A role for oxalic acid generation in ozone-induced signalization in Arabidopsis cells. *Plant, Cell & Environment* **36,** 569–578.

Yokawa K, Kagenishi T, Kawano T, Mancuso S, Baluška F. 2011b. Illumination of Arabidopsis roots induces immediate burst of ROS production. *Plant Signaling and Behavior* **6,** 1457–1461.

Yokawa K, Kagenishi T, Kawano T. 2011c. Prevention of oxidative DNA degradation by copper-binding peptides. *Bioscience, Biotechnology, and Biochemistry* **75,** 1377–1379.

Yokawa K, Kagenishi T, Kawano T. 2011a. Superoxide generation catalyzed by the ozone-inducible plant peptides analogous to prion octarepeat motif. *Plant Signaling and Behavior* **6,** 477–482.